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Full Length Research Paper

Establishment of proteome spot profiles and comparative analysis of the red and green phenotypes of 'Bon Rouge' pear (*Pyrus communis* L.) leaves

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The 'Bon Rouge' pear (*Pyrus communis* L.) cultivar is characterized by high levels of anthocyanins, the pigments responsible for the red leaf and red fruit skin phenotype. Branches of 'Bon Rouge' pear trees planted in commercial orchards often revert to the original green phenotype. The study aimed at establishing proteome maps of 'Bon Rouge' pear leaves and at comparing the proteomes of the red and green phenotypes of these leaves. In this study, total proteins extracted from red and green leaves were resolved on two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) and stained with coomassie brilliant blue (CBB) to establish proteome maps. An average of 183 and 173 protein spots were detected in the red and green phenotypes, respectively. Using mass spectrometry, 12 differentially expressed proteins, as determined by the comparative PDQuest analysis of the two phenotypes, were positively identified. The identifications were then validated using the publicly available apple expressed sequence tag (EST) database. One of the proteins that was up regulated in the green phenotype was identified as phytochrome B, a protein involved with the inhibition of anthocyanin synthesis. Overall, the present data showed a predominant increase of photosynthesis-related proteins in the green leaves.

Key words: Pear, proteomics, anthocyanins, leaves, two-dimensional polyacrylamide gel electrophoresis.

INTRODUCTION

Pyrus communis L. and *Pyrus pyrifolia* (Burm.) Nak. (Japanese or 'nashi' pear) are the two main commercial pear species used in fruit production (Monte-Corvo et al., 2000). They represent the third most important fruit produced in temperate regions after grape (*Vitis vinifera* L.) and apple (*Malus* x *domestica* Borkh.), with over 19 million tons per annum (FAO, 2008). The species belong to the Rosaceae family, along with other economically important fruits like apple, peach (*Prunus persica* L.), apricot (*Prunus armeniaca* L.) and strawberry (*Fragaria* x *ananassa*). *P. communis* is widely cultivated throughout Europe, North and South America, Africa and Australia, while *P. pyrifolia* is mainly cultivated in Southern and Central China and Japan (Bell, 1990).

Plant colour determination is mainly produced by the accumulation of flavonoids (Winkel-Shirley, 2001). Anthocyanin pigments, the major flavonoid compounds are widely present in plants and are responsible for the red, purple or blue colouration of various organs. In flowers and fruits, anthocyanins provide colour to attract pollinators and seed-dispersing animals (Harborne, 1965; Winkel-Shirley, 2002). In photosynthetic tissues like leaves and stems, anthocyanins have been shown to protect cells from high light damages by decreasing the amount of radiation that reaches the photosynthetic system (Takahashi et al., 1991; Chalker-Scott, 2008). In addition, anthocyanins also act as powerful antioxidant molecules (Gould et al., 2002), making them appealing for human and animal nutrition.

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Originally derived from a bud mutation of 'William's Bon Chrétien' (Booi et al., 2005; du Preez et al., 2005), 'Bon Rouge' is characterized by red skinned fruits throughout the development stages and red leaves early in the season, while 'William's Bon Chrétien' pears are characterized by green skin that develops a red blush during maturation and green leaves throughout the growing season (Booi et al., 2005; du Preez et al., 2005). Branches of 'Bon Rouge' trees in commercial orchards often revert to the original 'Bon Chrétien' parent phenotype (Booi et al., 2005; du Preez et al., 2005), albeit coming from the same tree and exposed to the same environmental factors. A recent study revealed that the expression of anthocyanin synthase (*ANS*) and uridine 5'-diphospho glucose-flavonoid 3-*O*-glucosyltransferase (*UFGT*), two genes involved in anthocyanin biosynthesis, as well as of a myeloblastosis (Myb) transcription factor was higher in the red skin of 'Max Red Bartlett' pear fruits than in the yellow skin of 'Williams' fruits (Pierantoni et al., 2009). In apple, a Myb transcription factor has been reported as involved in the regulation of anthocyanin biosynthesis (Takos et al., 2006; Chagné et al., 2007; Espley et al., 2007). In grape, the insertion of a transposon is known to affect *UFGT* as well as the *VvmybA1* transcription factor, and in turn fruit pigmentation (Kobayashi et al., 2004). Comparative gene expression analysis between 'Bon Rouge' and its reverted branches suggested that the differences in anthocyanin content may result from the up regulation of genes associated with various stress responses (du Preez et al., 2005). However, the expression of the genes involved in anthocyanin biosynthesis did not fluctuate in the green phenotype, suggesting regulation at the post-transcriptional and/ or post-translational level (du Preez et al., 2005).

Proteomics, the systematic analysis of the protein population in a tissue, cell or subcellular compartment (van Wijk, 2001), can be used to link genotype and phenotype during plant growth and development (Zivy and de Vienne, 2000). Comparative proteome analyses are also employed to study differential protein expression in relation to genetic diversity of organisms (van Wijk, 2001). To date, only two proteomic analyses have been published in pear, both using fruits of the 'Conference' cultivar (Pedreschi et al., 2007, 2008). These papers aimed at analysing the core breakdown disorder, which occurs during storage (Pedreschi et al., 2007), and the physiological implications of four different storage conditions (Pedreschi et al., 2008).

In order to gain insight on pear leaf proteome as well as on the influence of leaf pigmentation on the proteome patterns, this work reports the establishment of the first pear leaf proteome maps. On average, 183 and 173 protein spots were visualized in the red and green phenotypes, respectively. To characterise the pear leaf colour change, the proteome maps of the red and green phenotypes were comparatively analysed using the PDQuest software. A total of eight of these differentially expressed proteins were identified, most of which are involved in photosynthesis.

MATERIALS AND METHODS

Plant materials

Leaves from red and green-reverted branches of 'Bon Rouge' pear trees cultivated in open field conditions at the Agricultural Research Council 'Bien Donné' experimental farm (Simondium, South Africa) were collected 15 to 30 days after blooming. A total of four trees per phenotype were used in this study. For each sample, five leaves from a single tree were bulked, rinsed with dH_2O, frozen in liquid nitrogen and transported to the laboratory for storage at - 80°C until use in protein extraction.

Protein extraction

Approximately 1 g of leaf material from the five collected leaves per tree was ground to a fine powder in liquid nitrogen with mortar and pestle and precipitated with 1 ml of 10% (w/v) trichloroacetic acid in acetone. Insoluble plant debris and precipitated proteins were collected by centrifugation at 13,200 × *g* for 10 min at 4°C. The pellet was washed three times with 500 µl of 80% (v/v) ice-cold acetone. After centrifugation at 13,200 × *g* for 10 min at 4°C, the collected pellet was then air-dried and resuspended in 300 µl of urea buffer [9 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)] by incubating for 30 min on ice and vortexing overnight at 4°C. Proteins were collected by centrifugation at 13,200 × *g* for 10 min at 4°C. Protein content was estimated by Bradford assay (Bradford, 1976) with modifications as described by (Ndimba et al., 2003) using bovine serum albumin as standard. The quality of the protein extracts was evaluated by separation on a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) performed using the Mini-Protean® III electrophoresis cell (Bio-Rad, Hercules, CA, USA) in running buffer [0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS] and loading approximately 15 µg proteins per sample. Proteins were visualized by staining with Coomassie brilliant blue (CBB) and imaged with a PharosFX™ plus molecular imager scanner (Bio-Rad).

Protein separation by two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Protein samples (150 µg) were mixed separately with 0.8% (w/v) dithiothreitol (DTT), 0.2% (v/v) ampholytes, pH range 3 - 10 (Bio-Rad), 0.002% (w/v) bromophenol blue to a final volume of 125 µl in urea buffer. Samples were then used to passively rehydrate linear 7 cm immobilized pH gradient (IPG) strips, pH range 4 - 7 (Bio-Rad) overnight at room temperature. The strips were subjected to isoelectric focusing (IEF) as the first dimension using the Ettan™ IPGphor II™ (GE Healthcare, Uppsala, Sweden Amersham, UK), in a stepwise programme for a total of 6,700 h at 20°C. Prior to the second dimension, strips were equilibrated twice for 15 min each with gentle shaking in an equilibration buffer (6 M urea, 2% (w/v) SDS, 0.05 M Tris-HCl, pH 8.8, 20% (v/v) glycerol, 0.002% bromophenol blue) firstly containing 1% (w/v) DTT and then 2.5% (w/v) iodoacetamide. Strips were then loaded on top of 12% SDS-PAGE gels, sealed with a layer of agarose gel [0.5% (w/v) agarose, 0.002% (w/v) bromophenol blue dissolved in running buffer] and electrophoresed in running buffer at 100 V until the bromophenol blue dye reached the bottom of the gel (about 90 min). Proteins were visualized by staining with CBB and imaged with a PharosFX™ plus molecular imager scanner (Bio-Rad).

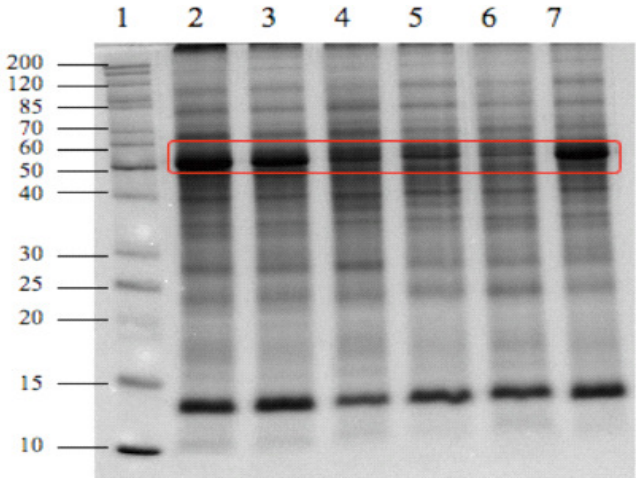

Figure 1. 1D-PAGE from the red and green phenotypes of 'Bon Rouge' leaves. Proteins (15 µg) were separated by 1D-PAGE and visualized by CBB staining. The protein profiles of three representative samples are shown in this figure. Lane 1: Molecular marker; lanes 2, 4 and 6: 'Bon Rouge' green phenotype; lanes 3, 5 and 7: 'Bon Rouge' red phenotype.

Gel analysis

Comparative analysis of 2D-PAGE from the red and the green phenotypes was carried out using the PDQuest 8.0.1 software (Bio-Rad). Four well-separated gels of each physiological state were used to create 'replicate groups' and a 'master gel' consisting of all the gels combined. To minimize experimental variations, spot intensity was normalized using the linear regression model on the basis of the total integrated optical density. After automated detection and matching of spots, manual editing was performed for every identified spots. Statistical, quantitative and qualitative 'analysis sets' were created by comparing the two phenotypes. In the statistic set, the student T-test with the significance level of 95% was selected. In the quantitative set, the upper and lower limits were set to 1.5 and 0.65, respectively. In the qualitative set, the detection limit of a spot versus background was set to 10-fold. Then, the Boolean analysis sets were created between the statistic sets and the quantitative or qualitative set. The spots from the Boolean sets were compared among the four biological replicates. Only spots displaying reproducible change patterns in at least two of the four replicate gels were considered as differentially expressed.

In-gel trypsin digestion

Spots of interest were excised manually and transferred into sterile microcentrifuge tubes. Gel pieces were washed twice with 500 µl of 50 mM ammonium bicarbonate for 5 min each time and a third time for 30 min, vortexing occasionally. The gel pieces were then destained twice with 500 µl of a solution containing 50% (v/v) 50 mM ammonium bicarbonate and 50% (v/v) acetonitrile for 30 min, vortexing occasionally. These gel pieces were dehydrated with 100 µl of 100% (v/v) acetonitrile for 5 min, and completely dessicated using a speed vac SC100 (ThermoSavant, Waltham, MA, USA). Proteins were in-gel digested with approximately 120 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) dissolved in 25 mM ammonium bicarbonate for 6 h at 37°C. Digestion was stopped by adding 50 µl of 1% (v/v) trifluoroacetic acid (TFA) and incubating for 2 h at room temperature before storage at 4°C until further analysis.

Protein identification by mass spectrometry (MS)

Prior to identification, samples were cleaned-up by reverse phase chromatography using ZipTip $_{C18}$ (Millipore, Billerica, MA, USA), pre-equilibrated first in 100% (v/v) acetonitrile and then in 0.1% (v/v) TFA and were eluted with 50% (v/v) acetonitrile. Digested proteins (1 µl) were mixed separately with the same volume of α-cyna-hydroxy-cinnamic (CHCA) matrix and spotted onto a target plate for analysis by matrix assisted laser desorption/ ionisation-time of flight (MALDI-TOF) MS using a Voyager DE Pro Biospectrometry workstation (Applied Biosystems, Forster City, CA, USA) to generate peptide mass fingerprinting (PMF). The MALDI-TOF MS was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential with optimized parameters. Close external calibration was employed using the Sequazyme calibration^{TM} mixture II containing angiotensin I, adrenocorticotropic hormone (ACTH; 1-17 clip), ACTH (18-39 clip) and bovine insulin (applied biosystems). This calibration method typically provided mass accuracy of 150 ppm across the mass range of 900 to 5,000 Da. Peptide spectra of accumulated 1,200 shots each were automatically processed for baseline correction, noise removal and peak deisotoping. Threshold was manually adjusted between 2 and 8% base peak intensity. All searches were performed against the national centre for biotechnology information non-redundant (NCBInr), mass spectrometry database (MSDB) and Swiss-Prot peptide mass databases using the MASCOT algorithm (<http://www.matrixscience.com/search_form_select.html). The searches were performed using a peptide mass tolerance of 100 ppm, allowing one missed cleavage and oxidation of methionine, as an amino acid modification. Candidate identifications with molecular weight search (MOWSE) scores higher than 85 were automatically considered as positive assignments. Other assignments with MOWSE score greater than 64 were considered positive if more than 10% of protein sequence was covered. If more than one protein satisfied the mentioned threshold criteria, the entry with the highest MOWSE score was assigned. Protein assignments were subsequently searched against the National Center for Biotechnology Information (NCBI) apple expressed sequence tag (EST) database using the full sequence and matched peptide sequences from any given assignment to determine similarity matches. Search criteria required the match of at least four of the matched peptides for validation. Identified proteins by Matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) were classified individually by their putative function as previously defined by (Bevan et al., 1998; Ndimba et al., 2005).

RESULTS AND DISCUSSION

One-dimensional expression profiles of pear leaf proteins

Total proteins extracted from pear leaves were firstly separated by one-dimensional (1D)-PAGE. Figure 1 shows the protein patterns for three representative samples of both the red and green phenotypes. This confirmed the equality of sample loadings and also showed that the protein band patterns were comparable between the two phenotypes, with the exception of a protein resolving at approximately 50 kDa. At this separation level, the abundance of this band showed variability among samples of the same phenotype but was generally more abundant in the 'Bon Rouge' green phenotype.

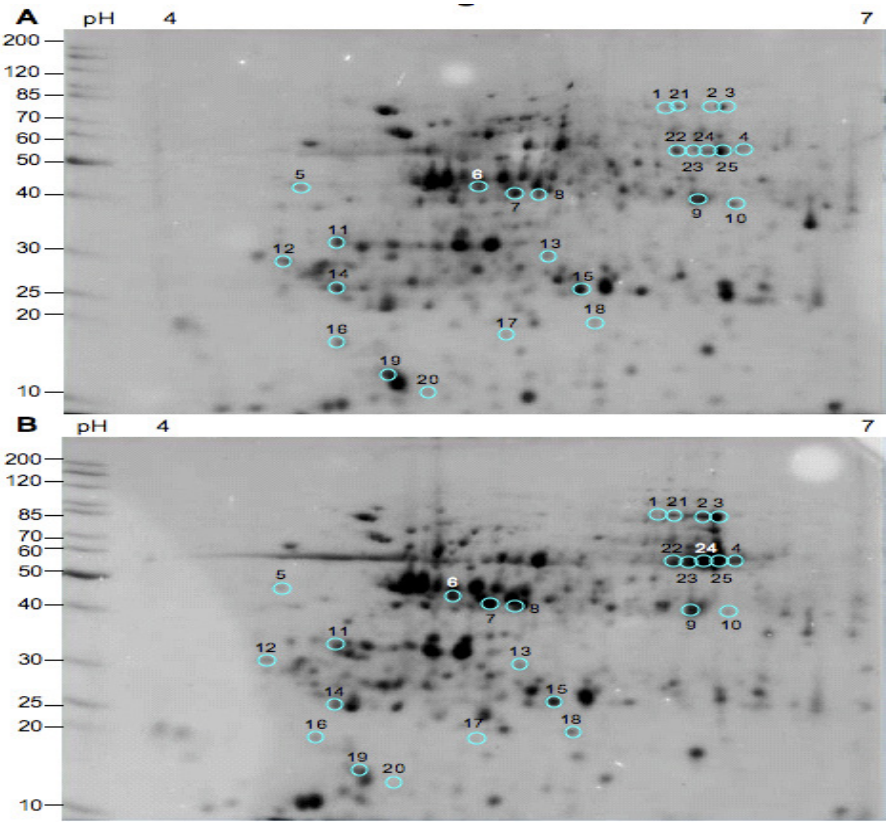

Figure 2. 2D-PAGE proteome maps from the (A) red and (B) green phenotypes of 'Bon Rouge' leaves. Proteins (150 µg) were separated using linear 7 cm IPG dry strips pH 4 - 7 in the first dimension and 12% SDS-PAGE in the second dimension. Protein spots were visualized by staining with CBB. Circles indicate proteins analysed by MALDI-TOF MS. Molecular masses of protein marker are shown on the left.

Development of proteome maps of 'Bon Rouge' pear leaf proteins

Pear leaf proteins were initially focused and resolved using pH range 3-10 IPG strips. Most of the visualized protein spots clustered in the region pH 4 - 7 (data not shown), indicating that a narrower pH range was required for increasing the resolution and for facilitating downstream protein identification by MALDI-TOF MS. Following application of a narrower pH range (4 - 7), a good protein separation and resolution was obtained (Figure 2). Samples from four biological replicates were then separated by 2D-PAGE for both the red and green phenotypes. Using PDQuest, an average of 183 abundant spots were visualized in the red phenotype and173 spots in the green phenotype of 'Bon Rouge' after CBB staining.

Comparative analysis of the red and green phenotypes of 'Bon Rouge' leaves

To characterize the differences between the red and green phenotypes of 'Bon Rouge' leaves, a comparative analysis was carried out using the PDQuest software. The expression of eight protein spots were up regulated in the green phenotype (Figure 2, spots 1, 2, 3, 4, 6, 7, 9 and 18), while nine were down regulated (Figure 2, spots 5, 8, 10, 11, 12, 14, 15, 16 and 19), in comparison with the red phenotype. In addition, three spots were detected only in the red phenotype (Figure 2, spots 13, 17 and 20).

Identification of proteins in 'Bon Rouge' pear leaves

For the identification of proteins, the peptide masses of each sample obtained by MALDI-TOF MS were submitted to MASCOT and searched against the MSDB, NCBInr and Swiss-Prot databases. This resulted in the positive identification of eight protein spots (that is, with a MOWSE score greater than 64, as this was considered as statistically significant at $p < 0.05$), corresponding to six non-redundant proteins. Some spot digests produced poor MALDI-TOF MS spectra. The source of most of these digest were faint gel spots, which is due to low expression and/or low molecular weight.

Table 1 shows the summary of pear proteins identified

Table 1. Protein spots from 'Bon Rouge' leaves identified by MALDI-TOF MS.

Spot	Accession	Protein name and species	Observed MW (kDa)/p*I*	Predicted MW (kDa)/p*I*	MOWSE score	Peptide matched/ Coverage	Peptide matched/similarities *Malus* ESTs	Green phenotype
Photosynthesis								
2	gi|75304978	Ribulose 1,5-bisphosphate carboxylase (fragment) (*Moraea autumnalis*)	85/6.30	46.1/6.12	66	13/20%	9/96%	+
3	gi|75305261	Ribulose 1,5-bisphosphate carboxylase large subunit (fragment) (*Neorautanenia mitis*)	85/6.40	51.4/6.14	112	12/26%	11/93%	+
4	gi|75298699	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (fragment) (*Elatostema acuminatum*)	55/6.40	44.6/6.40	73	10/14%	10/100%	+
6	gi|322416	Ribulose-bisphosphate carboxylase activase (*Cucumis sativus*)	43/5.30	45.7/7.57	72	5/14%	4/99%	+
18	gi|75284540	Phytochrome B (fragment) (*Cleome hassleriana*)	19/5.80	115.7/5.56	79	15/20%	6/80%	+
21	gi|75307090	Ribulose-1.5-bisphosphate carboxylase/ oxygenase large subunit (*Prunus apetela*)	85/6.20	51.7/6.63	89	15/30%	8/93%	
22	gi|75280748	Ribulose 1,5-bisphosphate carboxylase large subunit (Fragment) (*Nothofagus discoidea*)	55/6.20	49.5/6.23	95	13/27%	8/94%	
23	gi|75279426	Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (Fragment) (*Oserya coulteriana*)	55/6.20	49.7/6.34	116	12/26%	7/94%	
24	gi|1352782	Ribulose 1,5-bisphosphate carboxylase large chain (*Fragaria ananassa*)	55/6.30	51.6/6.04	97	10/24%	6/97%	
25	gi|75312637	Ribulose 1,5-bisphosphate carboxylase large subunit (fragment) (*Hyacinthus orientalis*)	55/6.30	49.6/6.23	106	10/26%	6/96%	
Signal transduction/Resistance								
16	gi|75146935	S-locus receptor kinase (fragment) (*Brassica oleracea*)	15/4.70	50/8.90	66	7/17%	6/35%	-
DNA binding protein								
9	Gi|7008714	MAR-binding filament like protein 1 (*Nicotiana tabacum*)	39/6.20	82.1/5.41	71	18/30%	3/64%	+
TCA cycle								
10	gi|122197432	NAD-dependent malate dehydrogenase (*Prunus persica*)	39/6.40	35.5/6.60	73	8/33%	7/96%	-

Pear proteins were identified by MALDI-TOF MS and classified into functional classes. Spot number corresponds to labelling in Figures 2A and B. The table lists the accession number, protein name, species for identification, theoretical and observed molecular weights (kDa) and pI, MOWSE score, number of peptide matched and sequence coverage of the positive identification, number of peptide matched and similarities from BLAST results against *Malus x domestica* ESTs database. Up and down regulations in the red phenotype in comparison to the green phenotype, as shown by the comparative PDQuest analysis, are indicated by the '+' and '-' signs, respectively.

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Identified proteins were classified into four functional categories (Table 1). As expected, the largest functional class, with five proteins, were associated with photosynthesis (spots 2 - 4, 6, 18, 21 - 25), as this is a major metabolic process in leaves. The majority of these proteins were identified as ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO; spots 2 - 4 and 6). The other classes represented here included signal transduction/resistance (spots 16), DNA binding proteins (spot 9) and tricarboxylic acid (TCA) cycle (spot 10).

Validation of identified proteins using EST database

Of these eight proteins, only one (spot 10) was identified against plants belonging to the Rosaceae family. This low rate could be attributed to the limited data of complete genes or cDNAs in the databases. None of the genome of plant species from Rosaceae is fully sequenced to date, but sequencing efforts have been initiated for apple, strawberry and peach (Rees, personal communication). This therefore implies that the identification by PMF will greatly improve in the near future as apple and pear are closely related (Pierantoni et al., 2004).

Since the number of pear ESTs in NCBI is currently limited to only 606 entries, while in apple, it is approaching 325,000 entries; the apple EST database from NCBI was queried to confirm the validity of the protein identification results. Both the full sequence of the protein assignment and the sequence of its matched peptides, as obtained by MASCOT, were searched against the EST database. Preliminary queries were carried out using the sequences of this protein identified in Rosaceae (spot 10) to demonstrate the validity of this search method (Table 1). BLAST searches resulted in 93% identity (Table 1). The similarity did not reach 100% probably because of modifications either at the post-transcriptional or post-translational levels and since apple EST data were used. Additionally, the sequences of matched peptides were used to BLAST against the same EST database. Seven out of eight peptides matched to apple EST sequences. In accordance with the MASCOT results, the BLAST search method resulted in the same identification, demonstrating the utility of ESTs for Rosaceae in proteomics investigations.

All the proteins identified by PMF were then validated by querying the apple EST database. Since a minimum of four matching peptides are statistically necessary for a protein to qualify as a confident match during PMF (Pappin et al., 1993), all the proteins with at least four peptides from the matched peptide list (from MASCOT results) matching to ESTs of the same protein were considered as validated. For the majority of these validated proteins (85%), the BLAST searches reached a minimum of 80% identity, further confirming the usefulness of the strategy and thus the positive identification of the proteins. However, low similarities were observed for two proteins (spots 9 and 16). In the case of spot 9, this might be due to the relatively low molecular weight of 15 kDa, which generated a small number of peptides after trypsin digestion, as previously described by (Thiede et al., 2005). The limited data of ESTs in the Rosaceae database might also affect the validation, and thus explain that no apple protein homolog was detected in NCBI for spot 16. Finally, as this has been previously reported (Mooney and Thelen, 2004), the short length or poor sequence quality of raw EST entries may have hampered PMF investigations.

Differentially regulated proteins

Of the 20 differentially regulated proteins, eight were positively identified (Figure 2 and Table 1, spots 2, 3, 4, 6, 10, 11, 17 and 19). Five of these eight proteins were identified as involved in photosynthesis (spots 2, 3, 4, 6 and 18). Of significant importance, the abundance of phytochrome B was up regulated in the green phenotype (spot 18). Phytochrome enzymes belong to a family of photoreceptors regulating the expression of numerous light-responsive genes, thus influencing a range of developmental and physiological events in response to the changing light environment (Neff et al., 2000; Quail 2002a, b; Wang and Deng, 2003). Phytochrome B is a family member that contributes to the induction of hypocotyl elongation and the inhibition of anthocyanin synthesis in continuous far-red light (Casal et al., 1998), through a negative correlation to phytochrome A (Short, 1999). The higher expression of phytochrome B in the green phenotype thus appears to be directly connected to the lower anthocyanin content. In addition, it has previously been shown that total levels of phytochrome A were not altered by over expression of phytochrome B (Short, 1999). Further analyses at the molecular level are necessary to understand the factors influencing the higher expression of phytochrome B.

Three of the up regulated protein spots were identified as RuBisCO (spots 2 - 4), the most abundant protein in leaves and other green tissues. This enzyme catalyzes the first major step in carbon fixation, the pathway responsible for the conversion of CO_2 into organic

compound using light energy (Kellogg and Juliano, 1997). The molecular weight of two of these spots (spots 2 and 3) was identical but their isolectric point (p*I*) varied slightly. Other unidentified spots were aligned with spots 2 and 3 as well as spot 4. To verify if these protein spots were also isoforms of RuBisCO, they were also analysed by MALDI-TOF MS (Figure 2 and Table 1, spots 21, 22, 23, 24 and 25). These five spots were positively identified as RuBisCO. The spot alignments thus suggested the occurrence of post-translational modifications. Previous studies revealed that the large and small RuBisCO subunits can become phosphorylated on serine and threonine residues (Guitton and Mache, 1987) as well as on tyrosine residues (Fedina et al., 2008). The phosphorylation of the components of the RuBisCO complex is suggested to control the formation and activity of the complex itself (Aggarwal et al., 1993). Thus, the alignment of spots observed on the 2D-PAGE strongly suggests that the protein might also be phosphorylated at multiple positions in pear leaves. Since the expression of several of these RuBisCO isoforms (spots 2 - 4) were up regulated in the green phenotype, the enzyme and/or its phosphorylated state might be associated with or affected by leaf pigmentation. However, further work is necessary to confirm the phosphorylation status of the protein spots, and its influence on the phenotype.

One of the protein spot identified as RuBisCO activase (spot 4) was also detected as up regulated in the green-phenotype. RuBisCO activase is involved in the regulation of RuBisCO activity (Portis, 2002; Spreitzer and Salvucci, 2002). This suggests a positive correlation of the expression of RuBisCO and its activase. Taken together, the higher expression of RuBisCO and RuBisCO activase from the Calvin cycle suggests that they may be indirectly controlled by phytochrome B, which probably induced the down regulation of the anthocyanin biosynthesis.

Finally, the primary objective of this study was to generate proteome spot profiles of 'Bon Rouge' pear leaves. For this purpose, proteins were successfully extracted from leaves of both the red and green phenotype for downstream analysis by 2D-PAGE. As a result, the first 2D-PAGE proteome spot profiles of pear leaves showed that the majority of proteins were resolved in the region of pH 4-7.

The second objective of this study was to compare expression profiles between the red and green phenotypes. The comparative analysis revealed that eight and nine protein spots were up and down regulated in the green phenotype, respectively. In addition, three spots were only detected in the red phenotype. As expected when working with leaf experimental material, a large proportion of the identified proteins were associated with photosynthesis, some of which were differentially expressed. In accordance with the gene expression analysis (du Preez et al., 2005), none of the differentially expressed proteins identified here fall in the classical anthocyanin biosynthesis pathways. One possibility is that, the high expression of phytochrome B, through its action of the down regulation of anthocyanins, may have triggered the changes in the photosynthesis machinery activity. On the other hand, leaf pigmentation could be inducing changes in leaf cellular molecular networks, process, and other metabolic activities, as hinted by the diversity of differentially expressed protein classes.

It would thus be interesting to study the chloroplast density and to measure the rate of photosynthesis, to further characterize the effects of colour changes of 'Bon Rouge' pear leaves. Since the majority of differentially expressed proteins were involved in photosynthesis, it would be of interest to build up proteome maps from chloroplast extracts to further investigate the pigmentation variations between the two phenotypes.

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Abbreviations

2D-PAGE, Two dimensional polyacrylamide gel electrophoresis; **CBB,** Coomassie brilliant blue; **EST,** expressed sequence tag; **PMF,** peptide mass fingerprinting; ***ANS*****,** anthocyanin synthase; ***UFGT*****,** uridine 5'-diphospho glucose-flavonoid 3-*O*-glucosyltransferase; **Myb,** myeloblastosis; **dH_2O,** distilled water; **SDS,** sodium dodecyl sulphate; **IPG,** immobilized pH gradient ; **NCBInr,** National Centre for Biotechnology Information Non-Redundant; **MSDB,** mass spectrometry database; **MOWSE,** molecular weight search; **NCBI,** National Center for Biotechnology Information; **BLAST,** basic local alignment search tool; **RuBisCO,** ribulose-1, 5-bisphosphate carboxylase/oxygenase.

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